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Purinergic transmission in the rostral but not caudal medullary raphe contributes to the hypercapnia-induced ventilatory response in unanesthetized rats

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ABSTRACT

The medullary raphe (MR) is a putative central chemoreceptor site, contributing to hypercapnic respiratory responses elicited by changes in brain PCO_2/pH. Purinergic mechanisms in the central nervous system appear to contribute to central chemosensitivity. To further explore the role of P2 receptors within the rostral and caudal MR in relation to respiratory control in room air and hypercapnic conditions, we performed microinjections of PPADS, a non-selective P2X antagonist, in conscious rats. Microinjections of PPADS into the rostral or caudal MR produced no changes in the respiratory frequency, tidal volume and ventilation in room air condition. The ventilatory response to hypercapnia was attenuated after microinjection of PPADS into the rostral but not in the caudal MR when compared to the control group (vehicle microinjection). These data suggest that P2X receptors in the rostral MR contribute to the ventilatory response to CO_2, but do not participate in the tonic maintenance of ventilation under room air condition in conscious rats.

1. Introduction

It has been proposed that central chemoreception (CCR), the specialized property of detecting CO_2/pH changes within the brain, is a widely distributed function in the central nervous system and involves many sites (Nattie, 2000; Nattie and Li, 2009), such as the medullary raphe (MR) which includes raphe magnus (RMg), raphe pallidus (RPa), and raphe obscurus (ROb). It is well established, indeed, that serotoninergic (5-HT) MR neurons play an important role in CCR (Ray et al., 2011; Richerson, 2004). Of interest are the observations that there is heterogeneity in the MR function concerning CCR, when the rostral (RMg) and caudal (ROb) MR are compared (da Silva et al., 2011; Dias et al., 2008; Li et al., 2006). The rostral MR is of particular interest in CCR since it contains a very large percentage of serotonergic neurons (Gao and Mason, 2001) and there is physiological and anatomic evidence for its role in the control of respiration during baseline and hypercapnic conditions (Dias et al., 2007; Holtman et al., 1990; Hosogai et al., 1998). However, the mechanisms associated with the CCR in the MR are not fully understood.

It has been firmly established that ATP has an important role as a neuro- and gliotransmitter in the central nervous system, in addition to its known role as an intracellular energy source (Burnstock, 1997). Among its actions, there is increasing evidence that ATP is an important mediator of CCR (Funk, 2010). Consistent with this possibility, the microinjection of suramin, a P2 receptor antagonist, into the medullary ventral respiratory column (VRC), attenuated respiratory responses to hypercapnia in anesthetized rats (Thomas et al., 1999). Moreover, the blockade of ATP receptors in the same region blocked the CO_2-evoked increase in frequency discharge of respiratory neurons (Thomas and Spyer, 2000). There is compelling evidence that the source of ATP in medullary VRC may be glial cells, which sense changes in the CO_2/pH, and thus release ATP to activate nearby neurons by a P2-receptor-dependent mechanism (Gourine et al., 2010; Wenker et al., 2010). However, the involvement of medullary raphe purinergic neurotransmission in the CCR has not been evaluated.

Several subtypes of P2X (ligand-gated cationic channels) and P2Y (G protein-coupled receptors) receptors have been cloned and described (North, 2002; Ralevic and Burnstock, 1998). P2X receptors have been found to be pH sensitive (King et al., 1996) and therefore could be implicated in the CCR by medullary neurons that express these receptors. Indeed, there is evidence supporting the hypothesis that ATP-P2X signalling has a functional role in the control of respiration and CCR. Moreover, P2X receptors are found in brainstem regions involved in respiratory control including the nucleus tractus solitarii (NTS), ventrolateral medulla (VLM), locus coeruleus (LC) and MR (Close et al., 2009; Gourine et al., 2003;

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Kanjhan et al., 1999; Yao et al., 2000). With respect to CCR, there is evidence that the chemosensitivity of neurons in the pre-Bötzinger Complex is inhibited by PPADS, a non-selective P2X antagonist (Thomas and Spyer, 2000). Considering the MR, an earlier study in anesthetized rats showed that microinjection of ATP in RMg and RPa produced inhibition or facilitation of respiration respectively, while the microinjection of PPADS had no effect on respiratory activity but partially blocked the ATP effects (Cao and Song, 2007). Nevertheless, the role of P2X receptors within the MR in CCR has not been explored in conscious animals.

Therefore, in the present study we evaluated, in different antero-posterior aspects of MR (rostral and caudal) of conscious rats, the role of P2X receptors on the respiratory responses to hypercapnia (7% CO_2). To this end, we performed experiments in unanesthetized rats, in which PPADS was microinjected into the rostral or caudal MR and respiratory parameters measured in room air and hypercapnia conditions.

2. Materials and methods

2.1. Animals

Experiments were performed on unanesthetized adult male Wistar rats weighing 270–300 g. The animals had free access to water and food and were housed in a temperature-controlled chamber at 24–25 °C (model: ALE 9902001; Alesco Ltda., Monte Mor, SP, Brazil), with a 12:12 h light–dark cycle (lights on at 7 AM). All experiments were performed in the light phase between 9:00 AM and 4:00 PM. Animal care was carried out in compliance with the guidelines set by SBCAL (Sociedade Brasileira de Ciência em Animais de Laboratório/Brazilian Society of Animal Lab Science) and with the approval of the University of São Paulo Animal Care and Use Committee (protocol no. 040/2007).

2.2. Surgery

Animals were anesthetized by administration of ketamine (100 mg kg^{-1}; i.p.) and xylazine (15 mg kg^{-1}; i.m.). The head and a portion of the abdomen were shaved, the skin was sterilized with betadine solution and alcohol and the animals were placed in a stereotaxic apparatus (insight, Brazil). Once fixed in the stereotaxic frame, rats were implanted with a stainless steel guide cannula. The guide cannula (0.7 mm o.d. and 15 mm in length) was implanted 3 mm above the rostral MR, which includes the RMg and RPa (10.52 mm caudal from bregma, in the midline, and 7.5 mm below the surface of the skull), or the caudal MR, which comprises the ROb (12.0 mm caudal from the bregma, in the midline, and 7.5 mm below the surface of the skull) (Paxinos and Watson, 1998). The cannula was attached to the bone with stainless steel screws and acrylic cement. A tight-fitting stylet was kept inside the guide cannula to prevent occlusion. Additionally, animals of all groups were submitted to paramedian laparotomy for the insertion of a temperature datalogger for body temperature measurements (SubCue, Calgary, AB, Canada). Body temperature readings were acquired at 5 min intervals. At the end of surgery, rats received 0.2 mL (1,200,000 units) of benzyl-penicillin administered intramuscularly. Surgical procedures were performed over a period of approximately 40 min and experiments were initiated seven days after surgery.

2.3. Measurements of respiratory variables and body temperature

Respiratory variables were obtained by the whole body plesthymography method (Bartlett and Tenney, 1970). Unanesthetized rats were placed into a 3.9 L Plexiglas chamber at 25 °C and allowed to move freely while the chamber was flushed with humidified air or with a hypercapnic gas mixture containing 7% CO_2 and 21% O_2 and N_2 balance. During each measurement of respiratory variables, the inlet airflow was interrupted for a short period of time (~1 min) while the chamber remained closed. Pressure oscillations caused by respiration were detected by a differential transducer and then amplified (MLT141 spirometer, Power Lab, AdInstruments, NSW, Australia). Recordings were saved and analysed using the PowerLab software (AdInstruments, NSW, Australia). Volume calibration was performed during each measurement throughout the experiments by injecting a known air volume (1 mL) inside the chamber. Respiratory variables such as respiratory frequency (fR) and tidal volume (V_T) were calculated described by Malan (1973). Ventilation ($\dot{V}_E$) was calculated as the product of V_T and fR and presented at ambient barometric pressure, at body temperature, saturated with water vapour at this temperature (BTPS). Body temperature was measured using an i.p.-implanted temperature datalogger (SubCue Dataloggers, Canada).

2.4. Drugs

The P2X receptor antagonist pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid 4-sodium (PPADS, Sigma Chemical, St. Louis, MO, USA) (Lambrecht, 2000), was freshly dissolved in pyrogen-free sterile saline (154 mM NaCl), and sodium bicarbonate was added to adjust the pH to 7.4. The concentration of PPADS (0.02 M) used in this study was selected on the basis of previous reports (Cao and Song, 2007).

2.5. Microinjection

For microinjections, a 1 μL syringe (Hamilton, Reno, NV, USA) connected to a PE-10 tubing and to a thin needle injector (33 gauge) was prefilled with PPADS, and then the needle injector was inserted into the rostral or caudal MR accordingly. The average accuracy of the 1 μL syringe is within ±1% of nominal volume and precision (coefficient of variation) within 1%, measured at 80% of total scale volume. The rostral MR contains the RMg while the caudal MR comprises the ROb. Prior to microinjection, animals were gently held in order to insert the needle injector into position in the guide cannula and once in the right position, the injections were manually initiated after a 30 s delay without handling or restraint of the rats. Animals did not undergo multiple injections. Each animal received only one microinjection and each experimental group was composed of different animals. The needle used for microinjection was 3 mm longer than the guide cannula. All microinjections were made with a volume of 50 nL, and in order to avoid reflux, a minute was allowed before removing the injection needle from the guide cannula.

2.6. Experimental protocols

Each animal was individually placed in a Plexiglas chamber (3.9 L) and allowed to move freely while the chamber was flushed with humidified room air. Following a 30 min acclimatization period, measurements of respiratory variables were taken. Subsequently, rats received microinjections of vehicle (saline) or the P2X receptor antagonist, PPADS, into the rostral MR or caudal MR, and a hypercapnic gas mixture (7% CO_2, 21% O_2, N_2 balance) was flushed into the chamber for 30 min. Respiratory variables were measured at 5, 10, 20 and 30 min after initiating hypercapnic condition. Finally, rats were returned to a period of normocapnia. Alternatively, in order to test the effects of PPADS in the baseline respiratory variables, the same procedures were performed, but instead of hypercapnia animals were maintained in normoxic, normocapnic condition after drug injection. All gas conditions were administered by a flow metre gas-mixing pump (Cameron Instruments GF-3/MP). O_2 (Raytech quadralyser 224A) and CO_2 (Beckman

LB2) gas analysers were used to monitor gas composition inside the animal chamber for all experimental protocols. Each animal was used once and received only one injection of PPADS or vehicle. All recording experiments were carried out at ambient temperature (24.5 ± 0.5 °C).

2.7. Histology

Upon completion of the experiments, animals were anesthetized with 2,2,2-tribromoethanol and perfused intracardially with saline followed by 4% paraformaldehyde. The brain was removed and stored in 4% paraformaldehyde for 4 h. Following fixation, paraformaldehyde solution was replaced for 20% saccharose (48 h, at 4 °C) to cryoprotect the tissue prior to processing. Tissue was frozen, sectioned on a cryostat at −20 °C (40 μm-thick coronal sections) and stained by the Nissl method for light microscopy. The location of injection was determined by the distance between the centre of injection and the caudal pole of facial nucleus (Paxinos and Watson, 1998). Only rats where the site of microinjection was located in the rostral and caudal aspect of the MR were considered for data analysis.

2.8. Statistical analysis

Values are reported as means ± SEM. $\dot{V}_E$, V_T and fR measurements were taken before CO_2 exposure, at 5, 10, 20 and 30 min during hypercapnia and after CO_2 exposure. Statistical analyses of the data were performed using a two-way ANOVA and Duncan's test for *post hoc* comparisons (Sigma Stat, Systat Software Inc., Point Richmond, CA, USA). Data was considered statistically significant when $p < 0.05$.

3. Results

Representative photomicrographs of typical sites of microinjections into the rostral MR and caudal MR are shown in Fig. 1A and B, respectively. In addition, diagrams of transverse sections of the brainstem showing the rostro-caudal distribution of microinjections sites are shown in Fig. 1C. These rostro-caudal sites are representative for all animals that received PPADS microinjections and underwent hypercapnic exposure protocol. Note in Fig. 1C that the rostral microinjections were located in the RMg nucleus ($n = 7$) and the caudal microinjections in the ROb nucleus ($n = 5$). For rostral MR (RMg) microinjection centre ranged from 10.5 to 11.58 mm caudal to bregma, while for caudal MR (ROb) microinjection ranged from 12.1 to 13.1 mm caudal to bregma.

3.1. Effects of PPADS microinjected into the rostral or caudal MR on baseline respiratory variables and body temperature

Fig. 2 summarizes data indicating that neither antagonism of P2X receptors (PPADS: 0.02 M; $n = 8$) nor microinjection of 50 nL of the vehicle (saline, 0.9% NaCl; $n = 7$) in the rostral or caudal MR changed baseline V_T, fR and $\dot{V}_E$ ($p > 0.05$) (Fig. 2, panels A–C). Data for rostral and caudal MR are plotted together in Fig. 2. Microinjection of PPADS into both rostral and caudal MR did not change body temperature compared with the vehicle group (37.4 ± 0.03 *vs.* 37.5 ± 0.04 ($p > 0.05$), respectively).

3.2. Effects of antagonism of P2X receptors on respiratory responses and body temperature to hypercapnia: rostral MR

Fig. 3 shows the effects of PPADS or saline microinjected into the rostral MR on, $\dot{V}_E$ (panel A), fR (panel B), and V_T (panel C) during 30 min of 7% hypercapnic exposure. Typical hypercapnia-induced hyperpnea was observed after saline microinjection ($n = 5$), whereas PPADS treatment ($n = 7$) attenuated that response at 5 ($p = 0.011$), 10 ($p = 0.02$), 20 ($p = 0.023$) and 30 min ($p = 0.016$) of hypercapnic exposure. The decrease in both V_T (Fig. 3C) and fR (Fig. 3B) were not significant ($p > 0.05$) after PPADS, but in conjunction they accounted for attenuated $\dot{V}_E$ (Fig. 3A). Microinjection of PPADS elicited a 34% and 32% attenuation of the ventilatory response to hypercapnia at 5 and 30 min (1857 ± 174 *vs.* 1412 ± 103 mL kg^{-1} min^{-1} at 5 min and 1882 ± 148 *vs.* 1468 ± 86 mL kg^{-1} min^{-1} at 30 min). 20 min after hypercapnia exposure, we did not observe a significant difference in the respiratory variables between the groups ($p > 0.05$). In addition, during hypercapnia exposure, no difference in body temperature was observed in rostral MR PPADS-treated animals compared with those in the vehicle group (36.7 ± 0.05 *vs.* 36.5 ± 0.4 ($p > 0.05$), respectively).

3.3. Effects of antagonism of P2X receptors on respiratory responses to hypercapnia: caudal MR

Microinjection of PPADS into the caudal MR had no effect on the respiratory responses to hypercapnia ($p > 0.05$). Fig. 4 shows the effect of PPADS microinjected into the caudal MR on $\dot{V}_E$ (panel A), fR (panel B), and V_T (panel C) during 7% hypercapnic exposure. Typical hypercapnia-induced increase in the respiratory variables was observed after saline microinjection ($n = 5$), but no change in these responses ($p > 0.05$) was observed in the group of animals treated with PPADS ($n = 5$) into the caudal MR (Fig. 4). As in the rostral PPADS injected group, there was no difference in body temperature between PPADS injected in the caudal MR group and the vehicle group (36.6 ± 0.04 *vs.* 36.5 ± 0.03 ($p > 0.05$), respectively).

4. Discussion

The present study provides evidence that P2X purinoceptors within the rostral, but not caudal MR, exert an excitatory modulation of the ventilatory response to hypercapnia in conscious rats. This is suggested since microinjection of PPADS, a broad spectrum P2X receptor antagonist, in the rostral MR, attenuated hyperpnea during 7% CO_2 exposure. The rostral aspect of MR includes the RMg whereas the caudal MR refers to the ROb nucleus. We chose to study these areas separately because it has been previously suggested that there is a heterogeneity in MR function with regard to respiratory control, when these rostral and caudal regions are compared (da Silva et al., 2011; Dias et al., 2008; Li et al., 2006). Chemical 5-HT neuronal lesion in the RMg (rostral MR) attenuated the hypercapnic ventilatory response by 31%, whereas the same chemical lesion in the ROb (caudal MR) reduced the hypercapnic ventilatory response by 12% (da Silva et al., 2011). While the rostral MR has been considered to be a chemosensitive site, the caudal MR apparently contributes to chemoreception indirectly, modulating respiratory control by interaction with other sites such as retrotrapezoid nucleus (RTN) (Dias et al., 2008; Li et al., 2006) and peripheral chemoreceptors (da Silva et al., 2011). Our results are very much in line with this notion since it was observed that PPADS affected the ventilatory response to CO_2 when microinjected within the rostral MR, but caused no change in ventilation when applied to the caudal MR.

The rostral MR has been extensively studied because it has been implicated in CCR (Bernard et al., 1996; Nattie and Li, 2001). Previous studies have shown that the neuronal pathway activated during hypercapnia includes the RMg (Teppema et al., 1997). In the present study, we have demonstrated that the antagonism of P2X receptors in the rostral MR caused a decreased ventilatory response to hypercapnia (Fig. 3). These results are consistent with the notion that ATP in the rostral MR has a role in chemoreception, but the phenotype

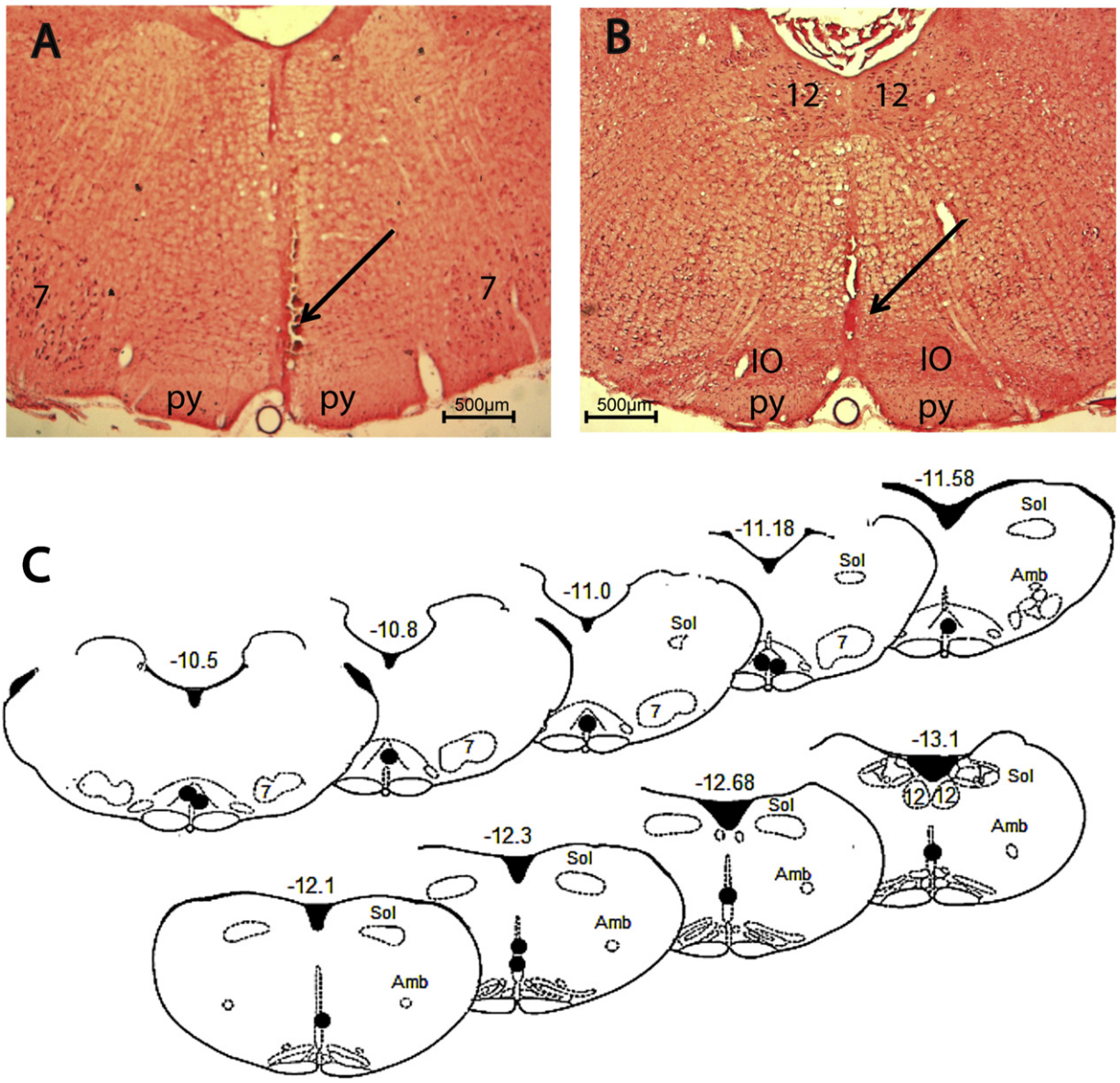

Fig. 1. Representative photomicrographs of brainstem coronal sections from two rats showing the centre of microinjections in the rostral MR (panel A) and caudal MR (panel B). Panel C shows a schematic drawing from rostral to caudal MR, modified from Paxinos and Watson (1998), illustrating the centre of microinjections (black filled circles) in each animal that underwent hypercapnic exposure. Note that rostral MR microinjections are located in the RMg nucleus (from 10.5 to 11.58 mm from bregma) and caudal MR microinjections are in the ROb nucleus (from 12.1 to 13.1 mm from bregma). Symbols: Amb, nucleus ambigus; py, parapyramidal tract; 4V, 4th ventricle; 7, facial nucleus; IO, olivary body; 12, hypoglossal nucleus; Sol, nucleus tractus solitarii.

of neurons involved in the ATP modulation of CCR is unknown. The neurons within the RMg are heterogeneous; however, the principal cell type is serotonergic, which has been proposed to be a central chemoreceptor (Ray et al., 2011; Richerson, 2004).

Given the primary role of the rostral MR 5-HT neurons in CCR and that there is evidence showing a significant degree of co-localization of purinergic receptors (including the subtypes: P2X, P2Y and P1) with tryptophan hydroxylase (TPH) immunoreactivity (a marker of 5-HT neurons) in the MR (Close et al., 2009), it is plausible that the attenuation of CO_2 ventilatory response may be *via* 5-HT neurons. However, the present study does not unveil this issue and it remains unknown whether ATP modulation of CCR in the rostral MR is effected through 5-HT neurons. Considering the P2X subtype, Close et al. (2009) have demonstrated that the percentage of purinergic receptor immunoreactive neurons that are TPH-positive is about 15%, whereas the percentage of TPH-positive neurons that are immunoreactive for purinergic receptors is about 64%. This suggests that there are other than 5-HT neurons which express P2X receptors and also that not all 5-HT neurons express this receptor. This raises the possibility that the CO_2-attenuated responses may involve other neuron phenotypes. Moreover, P2X receptors are also expressed in glia cells in other central nervous system regions (Dixon et al., 2004), which suggest that these cells may potentially contribute to ATP effects on the ventilatory response to the hypercapnia.

It has been suggested that P2X receptors are involved in the mechanisms underlying CCR. Purinergic transmission by neuronal P2X2 receptors is enhanced by acidotic conditions (King et al., 1996). Moreover, the chemosensitivity of respiratory neurons in the pre-Bötzinger complex is blocked by P2 receptor antagonists (Thomas et al., 1999). Presently, seven P2X types have been identified in mammals (North, 2002). PPADS has been shown to block P2X1-3, P2X2/3, P2X4 and P2X6 (Lambrecht, 2000; McLaren et al., 1994) and immunohistological studies have revealed moderate to

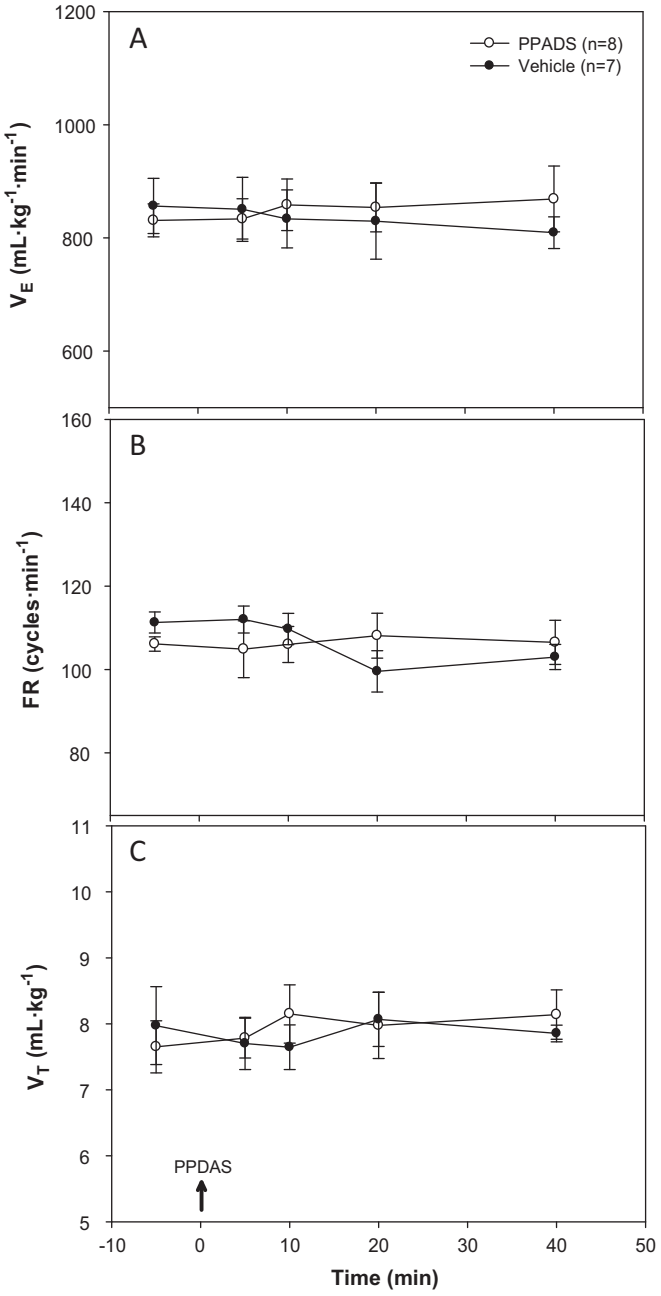

Fig. 2. Effects of microinjection of saline or PPADS (0.02 M) into medullary raphe (MR) on pulmonary ventilation ($\dot{V}_E$, panel A), respiratory frequency (fR, panel B) and tidal volume (V_T, panel C) of rats during normoxic normocapnic conditions (21% O_2, N_2 balance). The arrow indicates the time of injection. There was no effect of PPADS on basal respiratory parameters either in rostral or in the caudal MR microinjections. Data for rostral and caudal MR are plotted together. Values are expressed as mean ± S.E.M.

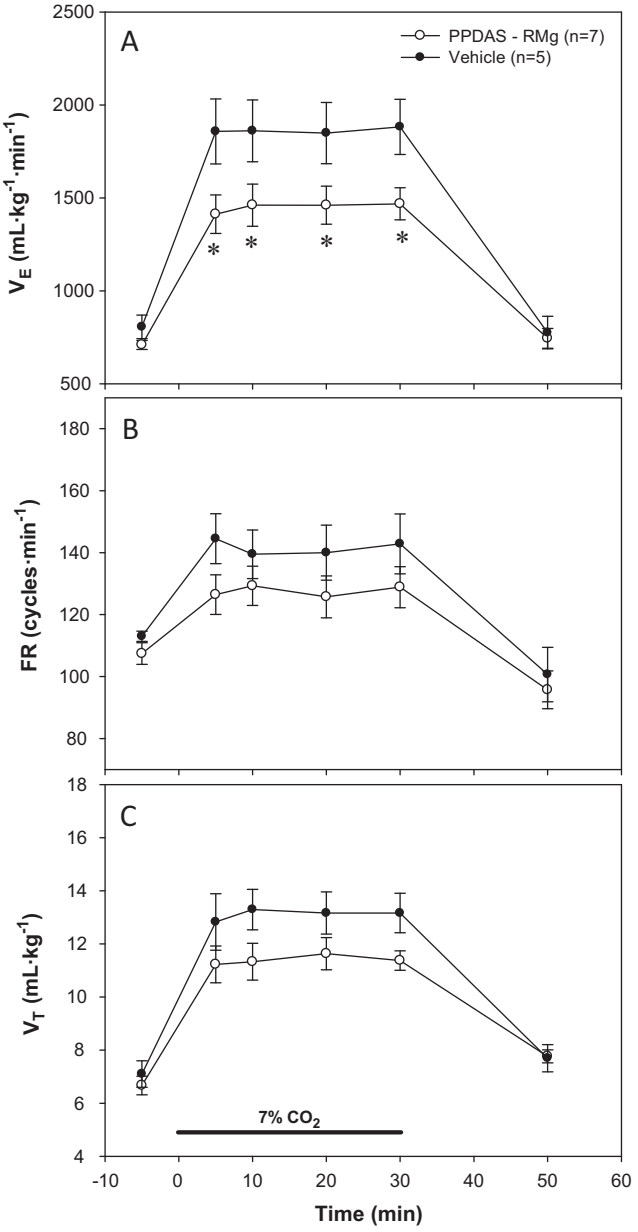

Fig. 3. Effects of microinjection of saline or PPADS (0.02 M) into rostral MR on pulmonary ventilation ($\dot{V}_E$, panel A), respiratory frequency (fR, panel B) and tidal volume (V_T, panel C) of rats exposed to hypercapnia (7% CO_2). PPADS attenuated the ventilatory response to CO_2. The symbol (*) indicates values that are significantly different comparing PPADS to saline ($p < 0.05$). Values are expressed as mean ± S.E.M.

high densities of P2X receptors in MR (Kanjhan et al., 1999; Yao et al., 2000, 2003), but the subtypes, within the rostral MR, responsible for the ATP-mediated modulation of hypercapnic chemoreflex, have yet to be elucidated. A prominent role for P2X2 receptors in central chemosensitivity has been suggested. Studies *in vitro* have shown that acidification of extracellular solution enhanced the ATP sensitivity of P2X2 receptor (King et al., 1996), while decreased the effect of ATP in cells expressing P2X1, P2X3 and P2X4 receptors (Stoop et al., 1997).

Our data provide support for the notion that ATP acting on P2X purinoceptors within the rostral MR plays a key role in modulation of CCR activation, but the source of ATP is still unclear. The literature has recently discussed the involvement of astrocytes in the control of pH-sensitive neurons (Gourine et al., 2010). Indeed, astrocytes have a favourable anatomic position, intimately associated with blood vessels supplying the lower brainstem (Gourine et al., 2010), which allows the close monitoring of the arterial blood composition entering the brain. Studies have demonstrated that glia have the ability to sense physiological changes in $PCO_2/[H^+]$ and convey this information to the respiratory neuronal network to change lung ventilation accordingly. Therefore it is reasonable to suggest that hypercapnia may elicit ATP release from astrocytes. The mechanisms involved in this release of ATP are still unknown. In the retrotrapezoid nucleus (RTN), it has been demonstrated that astrocytes release ATP in response to CO_2, and two mechanisms have been proposed. First, CO_2/pH elicits depolarization which causes

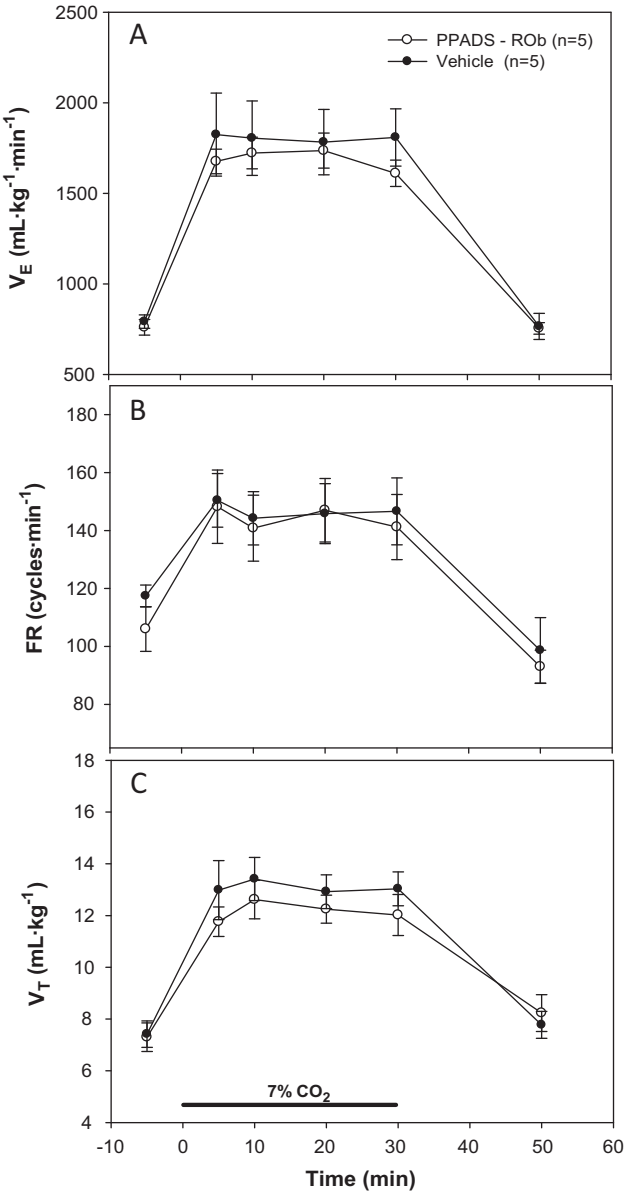

Fig. 4. Effects of microinjection of saline or PPADS (0.02 M) into caudal MR on pulmonary ventilation ($\dot{V}_E$, panel A), respiratory frequency (fR, panel B) and tidal volume (V_T, panel C) of rats exposed to hypercapnia (7% CO_2). PPADS had no effect on respiratory responses to CO_2. Values are expressed as mean ± S.E.M.

an increase in the intracellular levels of Ca^{2+} and subsequent ATP release by Ca^{2+}-dependent exocytosis (Gourine et al., 2010). The second mechanism consists of opening of Cx26 hemichannels that cause vesicle-independent ATP release (Huckstepp et al., 2010a, b; Wenker et al., 2010). At present it is unknown whether the mechanism underlying ATP release from astrocytes is shared between the MR and RTN.

In the present study, electroencephalographic or electromyographic data were not collected, so we cannot exclude the possibility that differences in arousal state between groups affected the results herein. However, we observed that the majority of our rats slept throughout most of the experimental period, with the exception of the beginning of the hypercapnic challenge when they were awake. Because this pattern was consistently observed in all groups, this should not affect the interpretation of the present data. Based on this methodological limitation, we also could not determine if the P2X receptors within the rostral MR have a differential role in hypercapnic chemoreflex according to arousal states.

In summary, the present data suggest that ATP, acting on P2X receptors in the rostral MR, exerts an excitatory modulation on the hypercapnic chemoreflex. No role is played by the P2X receptors in the caudal aspect of MR. Further investigations are needed to improve the current view of this system and the mechanisms involved in its physiological function.

Conflict of interest

There is no conflict of interest.

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